Molecular and biological characterization of *Toxoplasma gondii* isolates from free-range chickens from Guyana, South America, identified several unique and common parasite genotypes

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SUMMARY

The prevalence of *Toxoplasma gondii* in free-ranging chickens (*Gallus domesticus*) is a good indicator of the prevalence of *T. gondii* oocysts in the soil because chickens feed from the ground. The prevalence of *T. gondii* in 76 free-range chickens from Guyana, South America was determined. Antibodies to *T. gondii* were assayed by the modified agglutination test (MAT), and found in 50 (65·8%) of 76 chickens with titres of 1 : 5 in four, 1 : 10 in one, 1 : 20 in five, 1 : 40 in seven, 1 : 80 in six, 1 : 160 in eight, 1 : 320 in four, 1 : 640 or higher in 15. Hearts and brains of 26 chickens with titres of <1 : 5 were pooled in 5 batches and bioassayed in mice. Hearts and brains of 50 chickens with titres of 1 : 5 or higher were bioassayed in mice. *Toxoplasma gondii* was isolated by bioassay in mice from 35 chickens with MAT titres of 1 : 20 or higher. All mice inoculated with tissues of 30 infected chickens remained asymptomatic. *Toxoplasma gondii* isolates from 35 chickens were genotyped using 11 PCR-RFLP markers including SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, a new SAG2, and Apico. A total of 9 genotypes were identified, with 5 genotypes (nos 1, 4, 5, 6 and 7) unique to Guyana, 2 genotypes (nos 2 and 3) previously identified in chickens from Brazil, 1 genotype (no. 8) previously identified in chickens from Brazil, Costa Rica and Nicaragua, and 1 genotype (no. 9) belonging to the clonal type III lineage that exists globally. Infection with 2 genotypes was found from 1 chicken. This is the first report of genetic characterization of *T. gondii* isolates from any host from Guyana.

Key words: *Toxoplasma gondii*, chickens, *Gallus domesticus*, free-range, Guyana, genotype.

INTRODUCTION

Toxoplasma gondii infections are widely prevalent in human beings and other animals worldwide (Dubey and Beattie, 1988). Humans become infected post-natally by ingesting tissue cysts from undercooked meat, consuming food or drink contaminated with oocysts, or by accidentally ingesting oocysts from the environment. However, only a small percentage of exposed adult humans develop clinical signs.

Most *T. gondii* isolates from human and animal sources in Northern America and Europe have been grouped into 1 of 3 clonal lineages by multi-locus enzymes electrophoresis, PCR-RFLP and microsatellite typing (Dardé *et al*. 1992; Howe and Sibley, 1995; Ajzenberg *et al*. 2002*a*, *b*). Even though the difference among these 3 genotypes at the genome sequence level is less than 1%, they have strikingly different virulence phenotypes in mice, with type I strains uniformly lethal in outbred mice. In contrast, type II and III strains are significantly less virulent (Sibley and Boothroyd, 1992). It is of great interest to investigate whether the genotypes are related to the disease manifestations in human toxoplasmosis (Boothroyd and Grigg, 2002). Some studies reported that type I strains were more frequently associated in congenital and ocular toxoplasmosis in human patients based on one marker at the *SAG2* locus or a very limited number of genetic loci (Howe *et al*. 1997; Fuentes *et al*. 2001; Grigg *et al*. 2001; Aspinall *et al*. 2003; Kahn *et al*. 2005; Vallochi *et al*. 2005). Severe toxoplasmosis was reported in patients from French Guiana in association with atypical isolates of *T. gondii* (Dardé *et al*. 1998; Carme *et al*. 2002). However, we must be cautious

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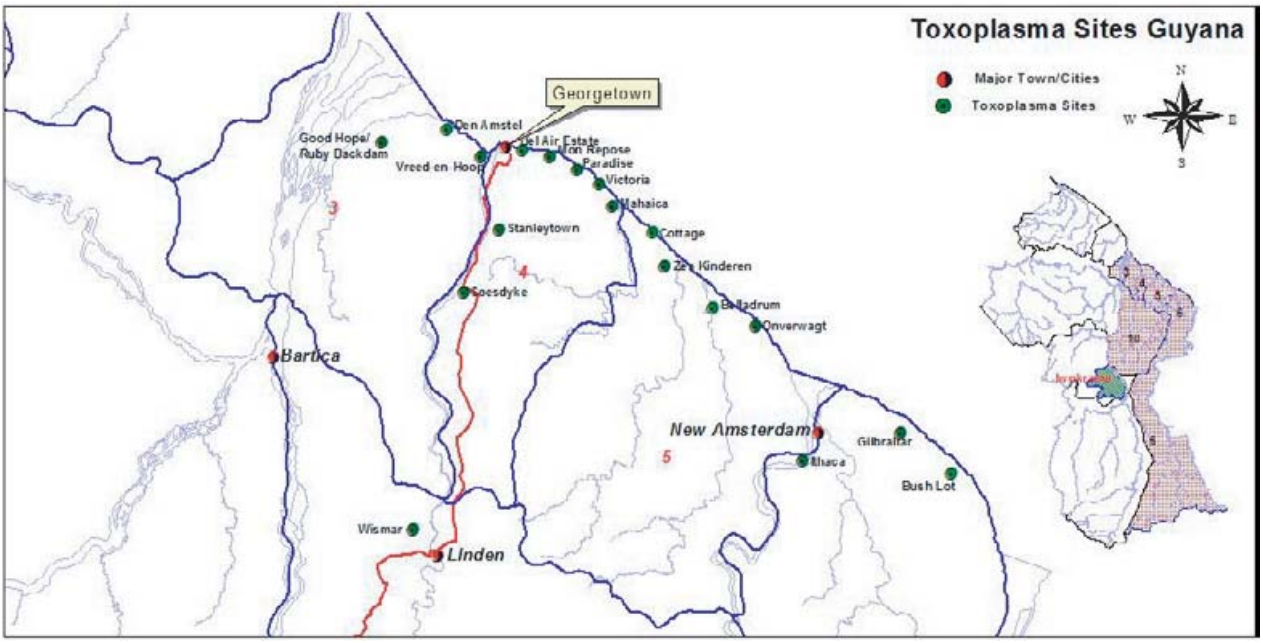

Fig. 1. Map of Guyana showing the collection sites.

about the interpretation of these studies, as the genetic markers used have limited power to distinguish parasite isolates (Su *et al*. 2006). For those *T. gondii* isolates that do belong to the same clonal lineage, biological information obtained from one representative may successfully predict the others because of their identical genetic background. The same may not be true for strains with different genetic makeup, particularly when frequent genetic crosses occur in the population. In such cases, there is low linkage disequilibrium and the association of genotypes with phenotypes is diminished (Su *et al*. 2006). One also needs to consider that genetic characterization of *T. gondii* isolates from humans is limited to isolates from patients ill with toxoplasmosis. To our knowledge there is no report of the genetic diversity of *T. gondii* isolates circulating in the general human population.

Recent studies on *T. gondii* populations in the animal populations started to reveal the diversity of the parasite. We have recently found that the isolates of *T. gondii* from Brazil are biologically and genetically different from those in North America and Europe (Dubey *et al*. 2002, 2007*a*; Lehmann *et al*. 2006). *Toxoplasma gondii* isolates from asymptomatic chickens from Brazil were more pathogenic to mice than isolates from Europe or North America, irrespective of the genotype (Dubey *et al*. 2006*a*). Additionally, most isolates from chickens from Brazil were not clonal (Dubey *et al*. 2007*a*). These worldwide studies in free-range chickens are important since chickens become infected mostly by feeding from ground contaminated with oocysts, and thus prevalence of *T. gondii* in chickens is a good indicator of the strains prevalent in their environment (Ruiz and Frenkel, 1980).

In the present study, we attempted to isolate and genotype *T. gondii* from chickens from Guyana, South America and compare the results with those obtained from other countries in South America.

MATERIALS AND METHODS

Naturally-infected chickens

During June, July, and August 2006 a total of 76 free-range chickens were obtained in 6 batches from 17 properties from various locations on coastal Guyana, with 2 collections being made 32 km (Soesdyke) and 92 km (Wismar) south of Georgetown, the latter being out of the coastal zone. Collection sites were separated from each other by a minimum of 6 km (Fig. 1).

Chickens were purchased, killed by cervical dislocation, and samples of brain, whole heart, and blood were collected from each chicken and kept at 4 °C until sent with cold packs by air to Beltsville, Maryland. Two to five days elapsed between killing of chickens and receipt of samples at Beltsville.

Serological examination

Sera of chickens were tested for *T. gondii* antibodies using 8 dilutions, from 1 : 5 to 1 : 640 with the modified agglutination test (MAT) as described by Dubey and Desmonts (1987).

Bioassay of chickens for T. gondii *infection*

Tissues of all chickens were bioassayed for *T. gondii* infection. Brains, and hearts of 50 chickens with

titres of 1 : 5 or higher were bioassayed individually in outbred female Swiss Webster mice obtained from Taconic Farms, Germantown, New York, as described (Dubey *et al*. 2002). Brains and hearts from 26 seronegative (MAT <1 : 5) chickens were pooled for specimens in each batch (5, 8, 5, 6, 2 chickens in batches 1–5; all chickens were seropositive in the sixth batch and bioassayed in mice. Tissues were homogenized, digested in acidic pepsin, washed, and aliquots of homogenates inoculated subcutaneously into 4 mice (Dubey, 1998).

Mice that received chicken tissues were examined for *T. gondii* infection. Tissue imprints of lungs and brains of mice that died were examined for *T. gondii* tachyzoites or tissue cysts. Survivors were bled on day 41 post-inoculation (p.i.) and a 1 : 25 dilution of serum from each mouse was tested for *T. gondii* antibodies with the MAT. Mice were killed 43 days p.i. and brains of all mice were examined for tissue cysts as described (Dubey and Beattie, 1988). The inoculated mice were considered infected with *T. gondii* when tachyzoites or tissue cysts were found in tissues.

Genetic characterization for T. gondii

Toxoplasma gondii DNA was extracted from the tissues of all infected mice from each group and strain typing was initially performed using the PCR-RFLP markers SAG1, SAG2, SAG3, BTUB and GRA6. These 5 markers allow us to quickly characterize all samples and to identify potential mixed infection in chickens. One or two representative DNA extracts from mice infected with the same chicken sample were genotyped with 6 additional genetic markers including c22-8, c29-2, L358, PK1, a new SAG2 and Apico to further identify isolates with high resolution (Dubey *et al*. 2006*b*; Su *et al*. 2006).

RESULTS

Antibodies to *T. gondii* were found in 50 (65·8%) of 76 chickens with titres of 1 : 5 in four, 1 : 10 in one, 1 : 20 in five, 1 : 40 in seven, 1 : 80 in six, 1 : 160 in eight, 1 : 320 in four, 1 : 640 or higher in 15.

A total of 36 *T. gondii* isolates were obtained from tissues of 35 chickens with titres of 1 : 20 or higher (Table 1). These isolates were designated TgCkGy 1–36. Two isolates were obtained from chicken no. 90, from which 1 of the 4 mice infected with tissues of the chicken had mixed allele types at loci SAG3 and PK1, indicating potential mixed infection, and this isolate is designated as TgCkGy36 (Tables 1 and 2). Five of the 36 *T. gondii* isolates (TgCkGy 2, 21, 22, 29, 34) were mildly pathogenic for mice (Table 1). In 4 of these isolates only 6 of 16 infected mice died of toxoplasmosis. In only 1 of these isolates (TgCkGy 21) all 4 inoculated mice died of acute toxoplasmosis on day 23 after receiving chicken tissues (Table 1).

Genotyping of these 36 isolates with 11 markers revealed 9 genotype groups with isolate TgCkGy36 showing mixed infection (Tables 1 and 2). One third (12/36) of the isolates belong to genotype no. 1, which was widespread in the areas sampled. Genotype no. 9 has type III alleles at all loci and likely belongs to the clonal type III lineage. A few pairs of genotypes differ only at 1 locus, including genotype nos 2 and 3 at locus Apico, genotype nos 4 and 5 at locus PK1, and genotype nos 7 and 8 at locus Apico, indicating each pair of genotypes are closely related.

DISCUSSION

The present study found that *T. gondii* isolates from Guyana were biologically and genetically different from Brazilian chicken isolates, irrespective of the location in Brazil. Table 3 summarizes information on mouse pathogenicity of isolates from chickens from South American countries. Differences in murine pathogenicity of isolates from asymptomatic chickens from countries in South America are evident; isolates from Brazil and Colombia were more pathogenic than from neighbouring countries. Among these countries, extensive data are available only from Brazil. Mouse pathogenicity was not affected by region of Brazil; *T. gondii* strains from the very northern state (Pará) were similar phenotypically to strains from the southern state, Rio Grande do Sul.

Recent studies using 11 PCR-RFLP markers indicated that virtually all isolates from 2 widely separated regions (Pará and Rio Grande do Sul) of Brazil were genetically distinct and this diversity was also evident among isolates from Guyana in the present study. At present, no correlation between mouse pathogeniciy and *T. gondii* genotype is established, and this may due to the great plasticity of gene expression in the parasite. Furthermore, there is no clear evidence that mouse pathogenicity of *T. gondii* isolates has any correlation with pathogencity in domestic animals or humans. Therefore, the concept of virulence in *T. gondii* is limited to the mouse model.

A comparison with previously published genotyping data from South and Central American isolates including dogs (Dubey *et al*. 2007*b*, *c*), cats (Dubey *et al*. 2006*c*; Su *et al*. 2006) and chickens (Dubey *et al*. 2006*b*, *c*, *e*, 2007*a*) showed that genotype nos 1, 4, 5, 6 and 7 are unique in chickens from Guyana, whereas genotype nos 2, 3, 8 and 9 were more widely spread. Both genotype nos 2 and 3 were found in chickens from Pará in Brazil, a location close to Guyana (Dubey *et al*. 2007*a*). Genotype no. 8 was found in chickens from Costa Rica (Dubey *et al*.

Table 1. Isolation of *Toxoplasma gondii* from free-range chickens from Guyana, South America

Geographical location						Isolation in mice	Genotype		
Household	Area	Village	Latitude, longitude	Chicken no.	MAT titre	No. infected[a]	Isolate ID	SAG1, SAG2, SAG3, BTUB, GRA6	c22-8, c29-2, L358, PK1, new SAG2, Apico
A	West Coast Demerara	Vreed-en-Hoop	58 11 W, 06 49 N	2	160	4	TgCkGy1	I,III,III,I,III	III,III,III,I,III,III
				4	160	4 (2)[b]	TgCkGy2	I,III,I,I,III	II,III,III,I,III,III
B	West Coast Demerara	Dan Amstel	58 15 W, 06 50 N	9	640	4	TgCkGy3	I,III,I,I,III	II,III,III,I,III,III
				10	320	4	TgCkGy 4	I,III,III,I,III	III,III,III,I,III,III
C	Demerara River	Wismar	58 18 W, 06 00 N	22	160	4	TgCkGy5	I,III,I,I,III	II,III,III,I,III,III
				26	640	4	TgCkGy6	I,III,I,I,III	II,III,III,I,III,III
D	East Coast Dem	Victoria	57 58 W, 06 45 N	32	80	4	TgCkGy7	I,III,I,III,III	III,III,III,I,III,III
E	East Bank Dem. River	Soesdyke	58 14 W, 06 32 N	34	80	4	TgCkGy8	I,III,I,III,III	III,III,III,I,III,I
				35	160	4	TgCkGy9	I,III,I,I,III	II,III,III,I,III,III
				36	640	1	TgCkGy10	I,III,I,III,III	III,III,III,I,III,I
F	East Coast Dem	Mahaica	57 55 W, 06 41 N	37	640	4	TgCkGy11	I,III,I,III,III	III,III,III,I,III,III
				38	320	4	TgCkGy12	I,III,I,I,III	II,III,III,I,III,III
				41	640	4	TgCkGy13	I,III,I,III,III	III,III,III,I,III,III
				42	640	4	TgCkGy14	I,III,I,III,III	III,III,III,I,III,I
G	East Coast Dem	Mon Repos	58 03 W, 06 48 N	43	640	4	TgCkGy15	I,III,I,III,III	III,III,III,I,III,I
				45	160	4	TgCkGy16	I,III,I,I,III	II,III,III,I,III,III
				46	640	4	TgCkGy17	I,III,III,I,III	III,III,III,III,III,III,III
				47	160	3	TgCkGy18	I,III,III,I,III	III,III,III,III,III,III,III
H	East Coast Dem	Paradise	58 00 W, 06 46 N	51	80	4	TgCkGy19	I,III,I,I,III	II,III,III,I,III,III
				52	40	4	TgCkGy20	I,III,I,I,III	II,III,III,I,III,III
I	East Coast Dem	Cottage	57 50 W, 06 38 N	53	160	4 (4)[c]	TgCkGy21	I,III,III,III,III	III,III,III,III,III,III,III
				54	640	4 (1)[d]	TgCkGy22	I,III,III,III,III	III,III,III,III,III,III,III
J	East Coast Dem	Zes Kinderen	57 49 W, 06 34 N	58	640	4	TgCkGy23	I,III,III,III,III	III,III,III,III,III,III,I
				60	40	2	TgCkGy24	I,III,III,III,III	III,III,III,III,III,III,I
K	West Coast Berbice	Belladrum	57 42 W, 06 30 N	65	640	3	TgCkGy25	I,III,I,I,III	II,III,III,I,III,III
L	West Coast Berbice	Onverwagt	57 37 W, 06 25 N	70	320	4	TgCkGy26	II or III,III,III,III,III	III,III,III,III,III,III,III
				72	20	3	TgCkGy27	II or III,III,III,III,III	III,III,III,III,III,III,III
				73	320	3	TgCkGy28	I,III,I,I,III	II,III,III,I,III,III
M	West Bank Berbice River	Ithaca	57 33 W, 06 14 N	75	640	4 (1)[e]	TgCkGy29	I,III,III,I,III	III,III,III,I,III,III
				76	80	3	TgCkGy30	I,III,III,I,III	III,III,III,I,III,III
N	Corentyne	Bush Lot	57 16 W, 06 12 N	79	640	1	TgCkGy31	I,III,I,III,III	III,III,III,I,III,III
O	Corentyne	Gibraltar	57 24 W, 06 18 N	84	40	4	TgCkGy32	I,III,I,I,III	II,III,III,I,III,III
P	Georgetown	Georgetown area	58 09 W, 06 48 N	85	40	1	TgCkGy33	I,III,III,I,III	III,III,III,I,III,III
Q	West Coast Berbice	Bel Air Estate	57 34 W, 06 19 N	88	80	4 (2)[f]	TgCkGy34	I,III,III,III,III	I,III,III,III,III,III
				90[g]	40	3	TgCkGy35	I,III,I,III,III	III,III,III,I,III,I
				90[g]	40	1	TgCkGy 36	I,III,I and III,III,III	III,III,III,I and III,III,I

[a] Four mice were inoculated with tissues of each chicken.
[b] Two mice died on day 12 p.i.
[c] All mice died on day 23 p.i.
[d] One mouse died on day 23 p.i.
[e] One mouse died on day 17 p.i.
[f] Two mice died on days 19 and 24 p.i.
[g] Mixed infection seen in this chicken.

Table 2. Summary of genotyping of *Toxoplasma gondii* from chicken from Guyana

	Genetic markers											
Genotype	SAG1	SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	new SAG2	Apico	Isolate ID
Reference	I	I	I	I	I	I	I	I	I	I	I	RH88
Reference	II or III[a]	II	II	II	II	II	II	II	II	II	II	PTG
Reference	II or III	III	III	III	III	III	III	III	III	III	III	CTG
Reference	I	II	III	II	II	II	u-1	I	u-2	II	I	COUGAR
Reference	u-1	I	III	III	III	u-1	I	I	III	II	I	MAS
Reference	I	III	III	III	III	I	I	I	u-1	III	I	TgCatBr5
No. 1	I	III	I	I	III	II	III	III	I	III	III	TgCkGy2,3,5,6,9,12, 16,19,20,25,28,32
No. 2	I	III	I	III	III	III	III	III	I	III	III	TgCkGy7,11,13,31
No. 3	I	III	I	III	III	III	III	III	I	III	I	TgCkGy8,10,14, 15,35
No. 4	I	III	III	I	III	III	III	III	III	III	III	TgCkGy17,18
No. 5	I	III	III	I	III	III	III	III	I	III	III	TgCkGy1,4,29,30,33
No. 6	I	III	III	III	III	I	III	III	III	III	III	TgCkGy34
No. 7	I	III	III	III	III	III	III	III	III	III	III	TgCkGy21,22
No. 8	I	III	III	III	III	III	III	III	III	III	I	TgCkGy23,24
No. 9	II or III	III	III	III	III	III	III	III	III	III	III	TgCkGy26,27
Mixed	I	III	I and III	III	III	III	III	III	I and III	III	I	TgCkGy36

[a] Cannot distinguish II or III.

Table 3. Mouse mortality patterns of *Toxoplasma gondii* isolates from chickens from South America

	T. gondii isolates			
Source of chickens	Total no.	No. of mouse virulent (%)[a]	SAG2	Reference
Argentina				
Buenos Aires	17	2 (11·7)	Present	Dubey *et al.* (2005*c*)
La Plata	9	2 (22·2)	Present	Dubey *et al.* (2003*c*)
Brazil (state or area)				
Total[b]	251[b]	107 (42·7)[b]	Absent[b]	
Amazonas	24	10 (41·7)	Absent	Dubey *et al.* (2006*a*)
Pará	17	13 (76·5)	Absent	Dubey *et al.* (2007*a*)
Paraná	13	9 (69·3)	Absent	Dubey *et al.* (2003*b*)
Rio de Janeiro	67	50 (74·7)	Absent	Dubey *et al.* (2003*a*)
Rio Grande do Sul	19	6 (31·6)	Absent	Dubey *et al.* (2007*a*)
São Paulo	22	19 (86·4)	Absent	Dubey *et al.* (2002)
Chile	22	0	Present	Dubey *et al.* (2006*b*)
Colombia	24	16 (66·6)	Absent	Dubey *et al.* (2005*a*)
Guyana	36	1 (3)	Present	Present study
Peru	10	1 (10)	Absent	Dubey *et al.* (2004)
Venezuela	12	1 (8·3)	Present	Dubey *et al.* (2005*b*)

[a] 100% mortality in Swiss Webster mice inoculated with chicken tissues.
[b] Data applicable to Brazil.

2006*b*), Nicaragua (Dubey *et al.* 2006*e*) and Pará in Brazil (Dubey *et al.* 2007*a*), indicating that it is widespread in South and Central America. Genotype no. 9 (type III lineage), one of the 3 predominant clonal lineages found in North America and Europe (Dardé *et al.* 1992; Howe and Sibley, 1995; Ajzenberg *et al.* 2002*a*), was also found in chickens from Brazil (Dubey *et al.* 2007*a*), Chile (Dubey *et al.* 2006*c*), Costa Rica (Dubey *et al.* 2006*b*) and Nicaragua (Dubey *et al.* 2006*e*), and dogs from Brazil (Dubey *et al.* 2007*c*), confirming its global presence. In summary, *T. gondii* isolates in chickens from Guyana are highly diverse, with a variety of genotypes existing both locally and globally.

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